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PURIFICATION OF RAT LIVER MITOCHONDRIAL δ-AMINOLAEVULINATE SYNTHASE

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SUMMARY. Mitochondrial δ-aminolaevulinate synthase was purified from drug-induced adult rat liver and identified for the first time as a protein of minimum molecular weight 70,000. The enzyme was also identified in mitochondria by pulse-labelling and immunoprecipitation and shown to have a molecular weight of 70,000. The purified enzyme was degraded by papain to smaller forms of molecular weight about 56,000 with no loss of enzyme activity. *In vitro* translation experiments suggest that the enzyme is synthesized initially as a larger precursor of molecular weight 76,000.

Purification of rat liver mitochondrial δ-aminolaevulinate synthase [EC 2.3.1.37] has been attempted by several groups (3, 4, 5). Paterniti and Beattie (4) purified mitochondrial enzyme with a minimum molecular weight of 58,000 while Kikuchi and Hayashi (5) using an immunological approach suggested that the molecular weight of the mitochondrial enzyme was 45,000; this value was recently revised to 66,000 (6).

We have recently reported a new purification procedure which enabled isolation from chick embryo liver mitochondria of undegraded δ-aminolaevulinate synthase with a minimum molecular weight of 68,000 (1). A purification procedure reported previously by Whiting and Granick (2) had shown the enzyme to

Abbreviations: DDC, 3,5-diethoxycarbonyl-1,4-dihydrocollidine; SDS, sodium dodecylsulphate

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have a molecular weight of 49,000. However our studies revealed that this smaller form was a breakdown product and that the native enzyme was readily degraded by proteolytic action (1). In view of this finding the possibility existed that the rat mitochondrial δ-aminolaevulinate synthase previously identified by other workers may also be a degraded form. In this paper, we purify intact rat liver mitochondrial δ-aminolaevulinate synthase and show it can be proteolytically modified without loss of activity. We present evidence that it is made initially as a higher molecular weight precursor.

MATERIALS AND METHODS

3,5-Diethoxycarbonyl-1,4-dihydrocollidine (DDC) was purchased from Eastman Organic Chemicals, Rochester, New York.

δ-Aminolaevulinate synthase assays were carried out as described by Whiting and Granick (2). One unit of δ-aminolaevulinate synthase activity is defined as the amount of enzyme which gives one nmole of δ-aminolaevulinate in 60 min under standard assay conditions.

Sodium dodecylsulphate (SDS) polyacrylamide gel electrophoresis was performed by the method of Laemmli (7) using 10% acrylamide. Staining of gels was carried out as described by the method of Fairbanks *et al.* (8). Non-denaturing disc-gel electrophoresis was performed in 5% acrylamide slab gels using the Tris-glycine system of Davis (9) except that the running pH was lowered from 9.5 to 9.0.

Drug Treatment of Rats. Mitochondrial δ-aminolaevulinate synthase was increased using the porphyrinogenic drug DDC. Male Wistar rats (150 - 200 g) were starved for 24 h prior to administration of the drug (0.4 g/100 g body weight). DDC was administered in 4 ml as a suspension in 10% (v/v) tragacanth mucilage via a stomach tube and induction allowed to proceed for 16 h.

RNA Isolation. Total RNA was isolated from livers of drug-treated or untreated rats by the guanidine HCl method (10) and total poly(A)-rich RNA was obtained by affinity chromatography on poly(U)-Sepharose 4B. The poly(A)-rich RNA was fractionated on 5 - 25% sucrose density gradient containing 70% formamide, 1 mM Tris-HCl, pH 7.6, 1 mM EDTA (buffer A), after heat denaturation (45°, 5 min) in buffer A plus 0.1% SDS. RNA fractions were ethanol precipitated and fractions containing poly(A)-rich RNA of size greater than 18S were used for cell-free translation.

Cell-free Protein Synthesis. Poly(A)-rich RNA was translated in a wheat germ cell-free system (10) at 26°C for 60 min.

Immunoprecipitations of cell-free translation products were carried out by the procedure of Matsuura *et al.* (11).

Immunoprecipitated protein was analysed by SDS-polyacrylamide gel electrophoresis and fluorography as described by Laskey and Mills (12) but without preflashing.

Antibody to chick embryo mitochondrial δ-aminolaevulinate synthase was shown to cross-react with the rat mitochondrial enzyme; 100 µl of antiserum neutralized 30 units of enzyme activity. Analysis by Ouchterlony double diffusion gave a single precipitin line when antiserum reacted against purified rat liver mitochondrial δ-aminolaevulinate synthase or a crude liver mitochondrial extract from drug induced rats. No precipitin line was observed with non-immune serum or with a similar extract from control rats.

RESULTS AND DISCUSSION

We have recently described (1) a purification procedure for the isolation to homogeneity of chick embryo liver mitochondrial δ-aminolaevulinate synthase. An identical procedure has been used to purify liver mitochondrial δ-aminolaevulinate synthase from drug-induced rats. Briefly, mitochondria from drug-induced rat livers were converted to mitoplasts and a soluble extract from freeze-dried mitoplasts chromatographed on Sephacryl S-200 in high salt buffer. Enzyme was subjected to chromato-focusing on a column of Polybuffer Exchanger 94 (Pharmacia) and eluted with a polybuffer gradient of pH 8.6 - 6.6 with δ-amino-laevulinate synthase eluting at a pH of about 7.4. Finally, the enzyme was subjected to affinity chromatography on CoA-agarose in the presence of 100 mM glycine and eluted with 50 mM 5'-AMP. The enzyme was purified 750-fold to a specific activity of 9,790 units/mg protein, the highest yet reported for rat liver mito-chondrial enzyme (Table 1). Figure 1 shows the protein profile at each stage of the purification as analysed by SDS-poly-acrylamide gel electrophoresis. A major protein of molecular weight 70,000 was purified together with three other minor proteins of molecular weight of around 27,000. (Significantly, there was no protein visible in the molecular weight area of 50,000.)

To confirm that the protein of molecular weight 70,000 was δ-aminolaevulinate synthase, a sample of enzyme from the CoA-

TABLE 1 PURIFICATION OF δ-AMINOLAEVULINATE SYNTHASE FROM RAT LIVER MITOCHONDRIA

Step and Fraction	Volume (ml)	Total Protein (mg)	Total Activity (units)	Specific Activity (units/mg protein)	Yield %
Mitochondria (from 86 g liver)	20	2320	30,238	13	100
Mitoplasts	40	514	28,044	54	93
Extract from freeze-dried mitoplasts	12	294	25,496	87	84
Sephacryl S-200 chromatography	41.6	102	22,694	223	75
Chromatofocusing	37	3.2	21,710	6,800	72
CoA-agarose affinity chromatography	17.3	0.94	9,200	9,790	30

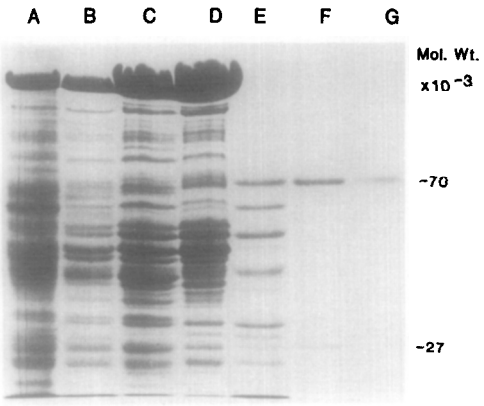

Figure 1. Protein profile at each stage of the purification as analysed by SDS-polyacrylamide gel electrophoresis. Equivalent amounts of δ-aminolaevulinate synthase activity were analysed from each stage of the purification. Lane A = mitochondria; Lane B = mitoplasts; Lane C = freeze-dried extract from mitoplasts; Lane D = Sephacryl S-200 chromatography; Lane E = chromatofocusing; Lane F = CoA-agarose affinity chromatography; Lane G = analysis of an enzyme active gel slice from a non-denaturing gel as described in text.

agarose column was extensively dialysed against 0.1 M triethylamine, and after freeze-drying was subjected to non-denaturing acrylamide gel electrophoresis. The gel was cut into slices which were assayed directly for δ-aminolaevulinate synthase activity and slices containing activity were analysed by SDS-polyacrylamide gel electrophoresis. These slices were shown by gel electrophoresis to each contain only 1 protein of molecular weight 70,000 (Figure 1, Track G) which co-migrated with the major purified band seen in Track F; this result established that the minimum molecular weight of rat liver mitochondrial δ-aminolaevulinate synthase is 70,000. This is apparently the intact form of the enzyme. Additional experiments were performed where drug treated or non-induced rats were pulse-labelled with [^{35}S]methionine for 45 min and mitochondrial extracts prepared by boiling in 3% SDS to minimize proteolytic action. Gel electrophoresis of the proteins showed a DDC inducible mitochondrial protein of minimum molecular weight 70,000 which

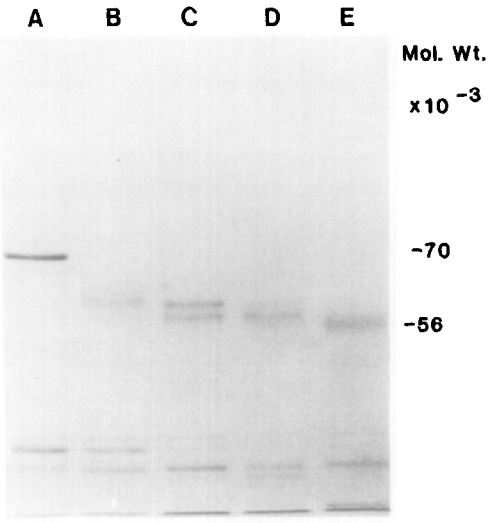

Figure 2. Papain treatment of purified δ-aminolaevulinate synthase. Aliquots (10 µg) of purified enzyme were incubated with varying amounts of papain at 37°C for 10 min. Antipain was added in a 20 molar excess over papain and samples were assayed for δ-aminolaevulinate synthase activity. The remainder was analysed by SDS-polyacrylamide gel electrophoresis: Lane A = untreated; Lane B = 1 µg papain; Lane C = 2 µg papain; Lane D = 20 µg papain and Lane E = 100 µg papain.

cross-reacted with antibody to pure chick embryo liver mitochondrial δ-aminolaevulinate synthase (result not shown).

We have previously shown (1) that chick embryo mitochondrial δ-aminolaevulinate synthase can be proteolytically modified without loss of enzyme activity. To investigate whether a similar situation existed for the rat enzyme, purified enzyme from the CoA-agarose column was incubated with papain. Analysis by SDS-polyacrylamide gel electrophoresis (Figure 2) showed that the δ-aminolaevulinate synthase of molecular weight 70,000 was progressively degraded by increasing amounts of papain to smaller forms of molecular weight about 56,000. (The lower molecular weight bands of about 27,000 were also digested.) However, there was no loss of δ-aminolaevulinate synthase activity following treatment of the enzyme with the different concentrations of papain.

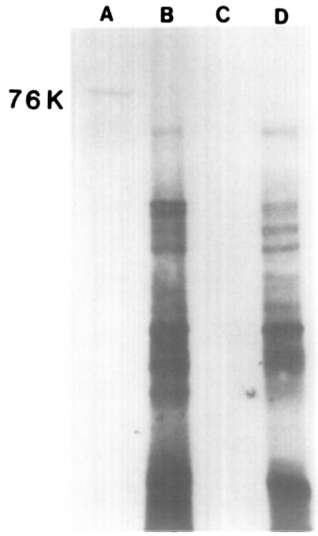

Figure 3. SDS-polyacrylamide gel electrophoresis of products from the translation of poly(A)-rich RNA in a wheat germ cell-free system. Poly(A)-rich RNA (0.5 µg) from induced and non-induced livers was translated *in vitro* in the presence of 0.5 µCi of [^{35}S]methionine (specific activity = 1200 Ci/mmol). Products were immunoprecipitated using chick anti-δ-amino-laevulinate synthase antibody. Lane A = immunoprecipitate from induced RNA; Lane B = total translation products from induced RNA; Lane C = immunoprecipitate from non-induced RNA; Lane D = total translation products from non-induced RNA.

Although the rat mitochondrial δ-aminolaevulinate synthase had a minimum molecular weight of 70,000 it is apparently synthesized initially as a larger precursor of minimum molecular weight 76,000. Fractionated total poly(A)-rich RNA isolated from drug induced and non-induced rat livers was translated in a wheat germ cell-free system. The translation products were subjected to immunoprecipitation with antibody to homogeneous chick embryo mitochondrial δ-aminolaevulinate synthase (see Materials and Methods) followed by SDS-polyacrylamide gel electrophoretic analysis. A major immunoprecipitated protein of molecular weight 76,000 was observed when induced poly(A)-rich RNA was translated (Figure 3, Track A) but not when non-induced poly(A)-rich RNA was translated (Figure 3, Track C). This result suggests that rat mitochondrial δ-aminolaevulinate is synthesized initially

as a larger precursor. This finding is similar to that for the chick embryo liver mitochondrial enzyme (1) and implies that δ-aminolaevulinate synthase like many other mitochondrial enzymes is processed during transfer into mitochondria.

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